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(54) Title: DNA CONSTRUCT FOR PROVIDING RNA THERAPY

(57) Abstract

A DNA construct capable of generating a relatively high copy number of therapeutic RNA molecules, including ribozymes and anti-sense RNAs, in target cells for effecting gene therapy. The construct and method of the invention are particularly useful in suppressing hapadnavirus infection and may be clinically effective in treating hepatitis B virus carriers.

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DNA CONSTRUCT FOR PROVIDING RNA THERAPY

Field of the Invention

The present invention relates to the field of gene therapy and in particular to a novel DNA construct capable of generating therapeutic RNA molecules in target cells, and to the use of such constructs in suppressing hepadnavirus infection and in providing selected anti-sense RNA therapy to patients who would be benefitted by such therapy.

Description of the Prior Art

Recent advances in recombinant DNA technology have brought the substantial promise of gene therapy nearer to realization. Techniques have now been developed that enable introduction of genetic material into mammalian cells via viral particles functioning in the ordinary course of infection. See: e.g., D. McCormick, Bio-Technology, 3:689-93(1985). Retroviruses have been reported to be especially useful as vectors for accomplishing gene insertion. W. Anderson, Science, 226:401-09(1984). This therapeutic approach typically involves introduction of the retroviral vector *ex vivo* into autologous cells from the site at which the therapy is to be directed, followed by reimplantation of the cells.

An alternative strategy for gene therapy involves the use of anti-sense RNA specifically to block the function of a gene. See: I. Herskowitz, Nature, 329:219-22(1987); and M. Innoue, Gene, 72:25-34(1988).

It has also been proposed to use highly sequence-specific endoribonucleases, commonly known as ribozymes, to achieve cleavage and inactivation of gene transcripts *in vivo*. According to this strategy, assuming knowledge of the transcribed sequence of the gene, it should be possible to target one or more

ribozymes against specific RNA transcripts, with expression of the ribozyme *in vivo* cleaving the transcript so as to inhibit expression of the corresponding gene. J. Haseloff *et al.*, *Nature*, *334*:585-91(1988). It is possible to design specific ribozymes, by altering a portion of its nucleotide sequence, to direct cleavage against a wide variety of target RNAs. Thus, ribozymes are capable of targeted destruction of specific cellular or viral RNAs.

Attempts to date to introduce into cells foreign DNA encoding a ribozyme have resulted in relatively low copy numbers of the ribozyme, thus raising concern about the prospective clinical efficacy of this approach to gene therapy.

Summary of the Invention

In accordance with the present invention, there is provided a unique DNA construct capable of generating in target cells therapeutic RNA molecules, including ribozymes and anti-sense RNA, in relatively high copy number of up to 100,000 copies or higher. The DNA construct of the invention comprises a first DNA segment which enables the construct to autonomously replicate in the form of a DNA molecule, a second DNA segment capable of generating the therapeutic RNA molecules and a third DNA segment which comprises the second DNA segment and which is capable of autonomously replicating in the form of an RNA molecule in the target cells.

According to a preferred embodiment, the construct of the invention includes as the third DNA segment multiple copies of a specific RNA satellite agent, known as hepatitis D virus, which upon replication in target cells, forms both identical and complementary RNA sequences. Inserted into this third

DNA segment, comprising more than one copy of the hepatitis D virus genome is the second DNA segment which encodes ribozymes or anti-sense RNA molecules which are thus expressed in the target cells. The DNA copy of the hepatitis D viral genome, including the ribozyme or anti-sense encoding insert, is incorporated in a conventional cloning/expression vector, preferably an SV40 vector, to form a DNA construct which is able to replicate autonomously in the form of a DNA molecule, and leads to HDV RNA transcripts which proceed with extensive RNA-directed RNA replication.

In clinical applications of the construct of the invention, the ribozyme or anti-sense RNA encoded by the construct will be directed against the RNA pregenome of hepatitis B virus. Administration of the resultant construct, in the form of virions packaged in the protein coat of hepatitis B virus, is expected to effectively suppress hepatitis B virus infection in chronic carriers thereof. Of course, the present invention has broader applicability, as it enables site specific delivery of other therapeutically effective RNAs, including other ribozymes and anti-sense RNA, as a means of providing RNA therapy.

Among the more notable advantages of the DNA construct of the invention are its relative high copy number, tissue specificity and self-limiting replication.

The DNA construct and method of treating hepatitis B virus infection in accordance with this invention should provide needed relief to the substantial number of HBV carriers, for whom there is no satisfactory treatment currently available, and thereby diminish their risk of developing liver cirrhosis and hepatocellular carcinoma.

Brief Description of the Drawings

Referring to the accompanying drawings:

FIG. 1 is an illustration of the structural features of unit length circular genomic (Fig. 1B) and antigenomic (Fig. 1C) HDV RNA and the mRNA (Fig. 1A) involved in synthesis of the delta antigen, in which are represented a self-cleavage site (o), a polyadenylation site (▬), a polyadenylation signal (□) and, upstream of the latter signal, an open reading frame (195 amino acids) for the delta antigen.

FIG. 2 is a representation of the predicted secondary structure of the catalytic loop of a ribozyme of 35 bases directed against the chloramphenicol acetyltransferase (CAT) gene, as encoded by the DNA construct of the invention, with the insertion occurring between bases 796 and 798 of the HDV-derived segments of the construct. The asterisks (*) indicate potential base pairing; the arrow indicates the site of RNA cleavage on the CAT gene. The 21-nucleotide upper strand is designated Sequence I.D. No. 1, and the 48-nucleotide lower strand is designated Sequence I.D. No. 2.

FIG. 3 is an audioradiograph, resulting from northern blot analysis of RNA recovered from cells transfected with a DNA construct of the invention, comprising two copies of an HDV genome modified in accordance with this invention to include the anti-CAT ribozyme represented in Fig. 2; the audioradiograph shows that the HDV genome, as modified, was capable of replication in target cells (Lane 2). The RNA undergoing analysis was recovered from transfected COS7 cells, comparing RNA from the same cells which were transfected with a construct comprising three DNA copies of wild-type HDV (Lane 1), a construct comprising two DNA copies of an HDV genome modified to include the anti-CAT ribozyme represented

in Fig. 2, a delta antigen-expressing clone (Lane 3), and untransfected cells (Lane 4; negative control). A positive control, i.e. RNA from the liver of an infected chimpanzee, and a molecular weight marker phiX/HaeIII, are also shown (Lanes c and M, respectively). The arrow indicates the mobility of unit-length genomic HDV RNA.

FIG. 4 is an audioradiograph resulting from a CAT mRNA assay via S1 nuclease; the mRNA was recovered from COS7 cells expressing CAT by activation of the CMV-IE promoter. Cells were transfected with (i) an HDV-containing plasmid vector pSVL, without the anti-CAT ribozyme insert (Lane 2), (ii) the same plasmid modified to include the anti-CAT ribozyme represented in Fig. 2 (Lane 3) and (iii) a construct comprising wild-type HDV (Lane 4). Molecular weight markers and an aliquot of undigested probe are also shown (Lanes M and p, respectively).

FIG. 5 is a representation of the anticipated secondary structure of the catalytic loop of a ribozyme of 35 bases, which is expected to have clinical application against conserved portions on the genomes of three hepadnaviruses (WHV, GSMV and HBV), as encoded by a DNA construct of the invention, with the insertion occurring between bases 795 and 796. The asterisks (*) indicate potential base-pairing; the arrow indicates the site of RNA cleavage on the hepadnavirus genome. The 20-nucleotide upper strand is designated Sequence I.D. No. 4, and the 55-nucleotide lower strand is designated Sequence I.D. No. 5.

Detailed Description

The following words and phrases are defined for reference in the description of invention as follows:

1. Therapeutic RNA molecule -- an RNA molecule that can be used intracellularly for a therapeutic purpose. For example, an RNA molecule that comprises a ribozyme, as discussed above, can be designed to bind and exert catalytic cleavage activity against a selected viral or cellular RNA. Additionally, an RNA sequence designed to be the complement of a selected viral or cellular RNA can bind and inactivate that RNA species. Both ribozymes and "anti-sense" RNA molecules can be of therapeutic value in the cell by inhibiting the expression of a detrimental gene product or by inhibiting the replication of a virus which relies on the function of an RNA species.

2. Autonomous replication -- the replication of a DNA or RNA molecule which is not dependent on the replication of other DNA or RNA molecules within a cell. Generally, DNA or RNA molecules may replicate autonomously if they possess an origin of replication, allowing cellular polymerases to recognize them as "replicable" species.

3. Identical and complementary RNA -- identical RNA strands comprise identical nucleotide sequences, while their complementary strands are related by means of Watson-Crick base-pairing.

4. Copy number -- the multiplicity per cell of RNA strands containing the therapeutic RNA.

The DNA construct of the invention comprises three segments. The first segment includes a means for autonomous replication of the entire DNA construct as a DNA molecule in appropriate host cells. This segment may be selected from any number of vectors which possess an origin of replication operable in the selected host cell. Suitable vectors include, for example, pBR322 plasmid derivatives to enable autonomous replication in *E. coli*, SV40-based plasmid

vectors to allow autonomous replication in selected animal cells, or "combination" vectors which would enable the construct to replicate in more than one type of host cell. In a preferred embodiment, a combination vector is used, namely, pSVL (Pharmacia), which carries pBR322 sequences as well as SV40 sequences, allowing the vector to be propagated in either E. coli or in certain cultured animal cells (e.g. COS monkey cells).

The DNA construct of the invention also contains a segment which is capable of replication as RNA in a suitable target cell. In general, such segments can be obtained as cDNA copies of selected viral RNA genomes containing an origin of replication. The process of obtaining cDNA copies from RNA through the use of reverse transcriptase is well known and commonly used by those skilled in the art.

An advantageous source of autonomously replicating RNA is the class of RNA virus-like agents known as "satellite RNAs." Hepatitis D virus is a member of this class of virus-like species. In a preferred embodiment, the hepatitis D virus genome is obtained from infected woodchuck hepatocytes and reverse-transcribed into cDNA molecules, which are then assembled to provide a cDNA copy of the full-length genome. The HDV cDNA is then inserted into the pSVL vector using conventional cloning techniques. One important feature of the construct of the invention is that two or more such HDV-derived cDNA inserts must be present in the construct in order to achieve the desired effect.

The construct, as described thus far, is capable of autonomous replication as a DNA molecule in its entirety in selected host cells. In addition, the HDV-derived cDNA segments are capable of autonomous replication as RNA molecules in selected target cells.

However, the construct is not of therapeutic utility until it is modified to include a DNA segment that will ultimately generate the therapeutic RNA molecule. This segment must be inserted in the RNA-derived portions of the construct to enable replication of the therapeutic RNA molecule as an RNA species. It is important to select an insertion site in the RNA-derived segment which does not disrupt any functional portion of that segment. In a preferred embodiment, the insertion is made between positions 795 and 798 in each of two woodchuck HDV-derived cDNA inserts in the construct. This position is advantageous in that it avoids the portions of the HDV genome involved in critical secondary structure formation, gene expression, and the origin of replication. The structure and organization of the HDV genome is shown in Fig. 1 A-C, along with the location of insertion of the DNA segment which encodes a therapeutic RNA molecule. The sequence and organization of the woodchuck HDV genome is described more fully in Kuo et al., _J. Virol._ 62: 1855-61 (1988) and Taylor, _Seminars in Virology_ 1: 135-41 (1990).

The therapeutic RNA-generating DNA insert may comprise for example, sequences encoding a ribozyme or an "anti-sense" RNA molecule directed against a specific RNA target. To design either type of insert, the nucleotide sequence of the target RNA must be known. A relevant structure of a ribozyme is that of Haseloff & Gerlach, _Nature_ 334: 585-91 (1988). For a review, see Cech, _J. Amer. Med. Assn._ 260: 3030-34 (Nov. 25, 1988).

Therapeutic RNA-generating sequences may be inserted into the RNA-derived portion of the construct in several ways. A preferred embodiment involves polymerase chain reaction (PCR) amplification of an oligonucleotide encoding a ribozyme directed against a

selected mRNA or viral RNA species. Specific examples include a ribozyme directed against messenger RNA encoding chloramphenicol acetyltransferase (CAT) (Haseloff & Gerlach, Nature 334: 585-91, 1988), and a ribozyme targeted to disrupt hepatitis B pre-genomic RNA. The structures of these ribozymes are shown schematically in Figures 2 (Sequence I.D. No. 2) and 5 (Sequence I.D. No. 4), respectively. A ribozyme directed against the pre-genomic RNA of hepatitis B virus is contemplated to be of great utility in suppressing hepatitis B viral replication in infected cells.

To achieve insertion, the ribozyme-encoding oligonucleotide insert is flanked on either side by sequences complementary to the region surrounding position 795-798 of the woodchuck HDV genome. The oligonucleotide is hybridized to the construct, then subjected to PCR amplification by methods well known in the art. The resulting population is comprised almost exclusively of constructs containing the oligonucleotide insert.

An alternative method for inserting sequences into the construct of the invention involves introducing a restriction site into an appropriate position on the RNA-derived segment by PCR-amplified oligonucleotide-directed mutagenesis as described above. Inserts encoding therapeutic RNA molecules can then be introduced into the construct by traditional recombinant DNA methods involving restriction cleavage and ligation.

When administered to target cells, the DNA construct of the invention is capable of delivering RNA-replicable segments encoding therapeutic RNA molecules. This is a distinct advantage over previous methods for delivering therapeutic RNA molecules because it enables the generation of a very high copy

number of therapeutic RNA molecules in each target cell. As noted above, a therapeutic RNA molecule delivered at low copy number is unlikely to provide a clinically useful effect. In contrast, the construct of the invention, when administered to target cells, can produce up to 100,000 or more copies of the therapeutic RNA molecule per cell. A ribozyme thus generated is capable of having an appreciable inhibitory effect on RNA species to which it is targeted. This effect is demonstrated in greater detail in Example 3 below.

The means by which such a high copy number may be achieved by using the HDV-derived construct arises from the replicative cycle of hepatitis D virus, as shown in Figure 1, and described more fully by Taylor, Seminars in Virology 1: 135-41 (1990). When a DNA construct comprising the HDV-derived cDNA segments is administered to a target cell, the HDV genomic RNA, including the therapeutic RNA insert, is formed within the cell by means of transcription. The HDV genome then replicates by forming a complete anti-genomic complementary RNA sequence. Both the genomic and anti-genomic RNA sequences are capable of continued rounds of replication, forming many copies of both HDV genomes and anti-genomes. However, it has been shown that many-fold more HDV genomes are formed than are HDV anti-genomes. Thus, the high copy number of replicated genomes in a cell is comprised mostly of the RNA species which is identical to the HDV genome. Since it is the genomic strand which has been designed to contain the therapeutic RNA molecule, a correspondingly high copy number of the therapeutic RNA is formed, resulting in a demonstrable and clinically effective quantity of therapeutic RNA molecules in each target cell.

The desired effect of a therapeutic RNA, such as the ribozyme directed against CAT mRNA, has been demonstrated by transfecting cultured cells with a DNA construct of the invention, as described in Example 3 below. However, the greater utility of this invention is contemplated to be in the clinical application of said DNA constructs. The unique aspects of the hepatitis D virus lifecycle will enable constructs of the invention to be delivered in vivo directly to hepatocytes. This is possible because hepatitis D virus is not capable of packaging and releasing itself from host cells. HDV relies on functions supplied by other hepadnaviruses to complete that portion of its lifecycle. For example, when hepatitis B virus (HBV) provides the necessary packaging functions, the HDV envelope will contain the same targeting signals as the HBV envelope, thus specifying its target cell range to hepatocytes.

Since it is possible to carry out these HBV-dependent packaging steps in vitro, the DNA construct of the invention may be used initially to transfect cultured cells containing the necessary HBV helper functions. The HDV-derived segments of the construct, containing the insert encoding the therapeutic RNA, will be transcribed into HDV genomes and antigenomes and amplified to a high copy number, as described earlier. These genomes can then be packaged and released as intact virions carrying HBV targeting signals. The virions, containing HDV genomes with therapeutic RNA inserts, can then be administered in a suitable pharmaceutical preparation to patients infected with hepadnaviruses. The virions will target and infect hepatocytes, the site of pre-existing hepadnavirus infection. Within these target cells the HDV genomes will be capable of replication, producing hundreds of thousands of copies

of the therapeutic RNA. If the therapeutic RNA is designed to be a ribozyme directed against pre-genomic or other HBV RNA species, the HBV life cycle will be interrupted, causing suppression of HBV-induced disease symptoms.

The following examples are provided to describe the invention in further detail. These examples are intended merely to illustrate and not to limit the invention. Unless otherwise specified, general cloning methods following procedures set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989).

EXAMPLE 1

Construction of a DNA vector containing two cDNA copies of the woodchuck hepatitis D viral genome.

Cloning

The RNA from the liver of an HDV infected woodchuck was used to create a cDNA library in lambda gt11. Total RNA from the liver of an infected woodchuck was isolated by extraction with guanidine isothiocyanate and purified by centrifugation to equilibrium in cesium chloride (Chirgwin et al., Biochemistry 18: 5294-99, 1979). The RNA was further purified by rate zonal centrifugation in a sucrose gradient to obtain a size class containing full length HDV RNA. Briefly, this RNA was converted with reverse transcriptase and random oligonucleotide primers (Taylor et al., Biochim. Biophys. Acta 442: 324-30, 1976) to double-stranded cDNA. After sizing, EcoRI linker was added and the product was inserted at the EcoRI site of lambda gt11. The library of clones obtained was initially screened with the subgenomic length delta clone pkD1 (Denniston et al., Science 232: 873-75, 1986). After further rounds of screening, selected clones were subjected to DNA

sequence analysis to determine the complete nucleotide sequence of the woodchuck HDV.

DNA Sequencing and Analysis

The nucleotide sequencing reactions were done by the dideoxy chain termination method of Sanger et al., Proc. Nat. Acad. Sci. USA 74: 5463-67 (1977), with modifications by Zagursky et al., Gene Anal. Tech. 2: 89-94 (1985) and Tabor et al., Proc. Nat. Acad. Sci. USA 84: 4767-71 (1987). Areas of uncertainty were confirmed by use of the modified Maxam-Gilbert method (Bencini et al., Biotechniques Jan./Feb. 1984, pp 4-5). Computer-based analysis of the obtained sequence information was carried out by using the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (Devereaux et al., Nuc. Acids Res. 12: 387-95, 1984).

Three of the selected clones were transferred to the plasmid pGem4Blue (Promega). Parts of these inserts were assembled onto a single plasmid, oriented relative to the EcoRI site at position 1427 of the 1679-base HDV genome, thereby constructing a full-length DNA copy of the HDV genome. Then by a strategy involving partial digestions and additional insertions, the total insert was raised to a trimer. This trimer was excised with BamHI and PvuII and force-cloned into the vector pSVL (Pharmacia) that had been cut with BamHI and SmaI. The resulting construct was designated pSVL[D3].

EXAMPLE 2

Modification of unit-length HDV sequences to provide segments encoding a ribozyme directed against mRNA for chloramphenicol acetyltransferase (CAT).

Addition of sequences encoding an anti-CAT ribozyme (Haselhoff & Gerlach, Nature 334: 585-91 (1988)) was accomplished by PCR-amplified

oliognucleotide-directed mutagenesis of a non-essential portion of the HDV genome-derived segments of the constructs. Position 797 of the woodchuck HDV genome was selected for mutagenesis. An oligonucleotide, comprising the anti-CAT ribozyme-encoding sequence flanked by sequences complementary to positions 789-796 and 798-802 of the HDV genome was synthesized using phosphoramidite chemistry (Sequence I.D. No. 2). A unit-length sequence was modified in this way, using PCR, with enzymes and procedures from Perkin-Elmer Co. The sequence was cloned, raised to a dimer, then transferred to pSVL and designated pSVL[D2-anti CAT].

EXAMPLE 3

Transfection of target cells with a construct of the invention and replication therefrom of HDV genomes containing therapeutic RNA molecules.

Transfection

As described in Examples 1 and 2 above, a dimer of the woodchuck HDV genomic sequence, each monomer containing a ribozyme insert, was assembled in the SV40-based vector pSVL. The construct was such that the RNA transcription, controlled by the SV40 late promotor, produced transcripts of genomic HDV RNA. This plasmid, designated pSVL[D2-anti CAT]; was transfected into a monkey kidney cell line, COS7, originally isolated by Gluzman, Cell 23: 175-82 (1981).

The DEAE-dextran transfection procedure was used as described by Cullen, Meth. Enz. 152: 684-704 (1987). The procedure was scaled down to use 100,000 cells in a 16 mm diameter well, and to apply only 120 ng DNA in 50 uL per well. After transfection the media was changed every second day until harvest. The cells were washed and removed by scraping, then the RNA

contained therein was extracted and subjected to Northern blot analysis, as described by Chen et al., Proc. Nat. Acad. Sci. USA 83: 8774-78 (1988).

Northern analysis of transfected cells verified that the therapeutic RNA-containing HDV genomes of the construct were able to undergo normal rounds of replication as RNA molecules, as shown and described in Fig. 3 of the drawings. Additionally, HDV replication was sufficiently high to yield effective quantities of the anti-CAT ribozyme contained therein, as described in Fig. 4. As shown, target cells expressing CAT were subjected to transfection by constructs containing either (1) pSVL alone, (2) a construct of the invention (pSVL[D2-anti CAT]) or (3) a construct containing a trimer of HDV-derived cDNAs, but without a ribozyme insert. Transfection with the construct of the invention resulted in a 3- to 5-fold inhibition of CAT activity in said target cells.

The present invention is not limited to the embodiments specifically described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A DNA construct for generating therapeutic RNA molecules in target cells, said construct comprising:

i) a first DNA segment which enables said construct to autonomously replicate in the form of a DNA molecule;

ii) a second DNA segment capable of generating said therapeutic RNA molecule; and

iii) a third DNA segment which comprises said second DNA segment, and which is capable of autonomously replicating in the form of an RNA molecule in said target cells.

2. The DNA construct of claim 1, wherein said first DNA segment is a vector derived from an animal virus.

3. The DNA construct of claim 2, wherein the animal virus DNA vector is SV40.

4. The DNA construct of claim 1, wherein said first segment is capable of replicating as DNA in animal cells and in bacterial cells.

5. The DNA construct of claim 4, wherein said first segment is the vector pSVL.

6. The DNA construct of claim 1 wherein replication of said third DNA segment in target cells results in the formation of identical and complementary RNA sequences.

7. The DNA construct of claim 1, wherein said third DNA segment comprises multiple copies of a genome derivable from an RNA satellite agent.

8. The DNA construct of claim 6, wherein said RNA satellite agent is Hepatitis D Virus.

9. The DNA construct of claim 8, wherein said second DNA segment is positioned so as not to disrupt essential functional portions of said third DNA segment.

10. The DNA construct of claim 9, wherein said first segment comprises pSVL, said third segment

comprises two DNA copies of the genome of Hepatitis D virus, and said second segment is positioned between nucleotides 795 and 798 on each copy of said HDV genome.

11. The DNA construct of claim 1, wherein said therapeutic RNA molecule generated by said second DNA segment is a ribozyme.

12. The DNA construct of claim 11, wherein said ribozyme is directed against a viral RNA species.

13. The DNA construct of claim 11, wherein said ribozyme is directed against hepadnavirus pre-genomic RNA.

14. The DNA construct of claim 13, wherein said hepadnavirus is Hepatitis B virus.

15. The DNA construct of claim 11, wherein said ribozyme is directed against a cellular RNA species.

16. The DNA construct of claim 1, wherein the therapeutic RNA molecule generated by said second DNA segment is an anti-sense RNA molecule.

17. The DNA construct of claim 16, wherein said anti-sense RNA molecule is directed against a viral RNA species.

18. The DNA construct of claim 16, wherein said anti-sense RNA molecule is directed against hepadnavirus pre-genomic RNA.

19. The DNA construct of claim 18, wherein said hepadnavirus is Hepatitis B virus.

20. The DNA construct of claim 16, wherein said anti-sense RNA is directed against a cellular RNA species.

21. The DNA construct of claim 1, wherein said third DNA segment comprising said second DNA segment is replicated to a copy number of at least 100,000 RNA molecules.

22. A method of suppressing Hepatitis B virus in carriers thereof, said method comprising administering to said carriers the RNA replication products of the DNA construct of claim 11 in such a

way as to generate a high copy number of said ribozyme directed against RNA species of said Hepatitis B virus.

23. A method of providing selected anti-sense RNA therapy to patients, said method comprising administering to said patients the RNA replication products of the DNA construct of claim 16 in such a way as to generate a high copy number of said anti-sense RNA directed against a selected RNA species.

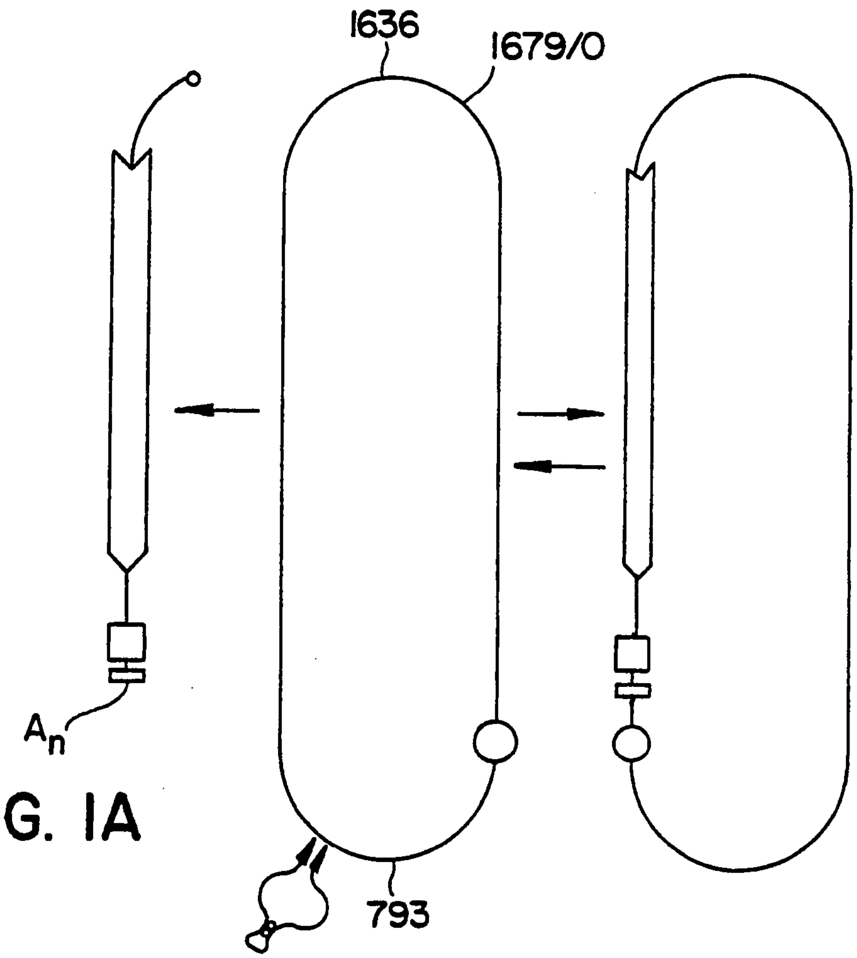
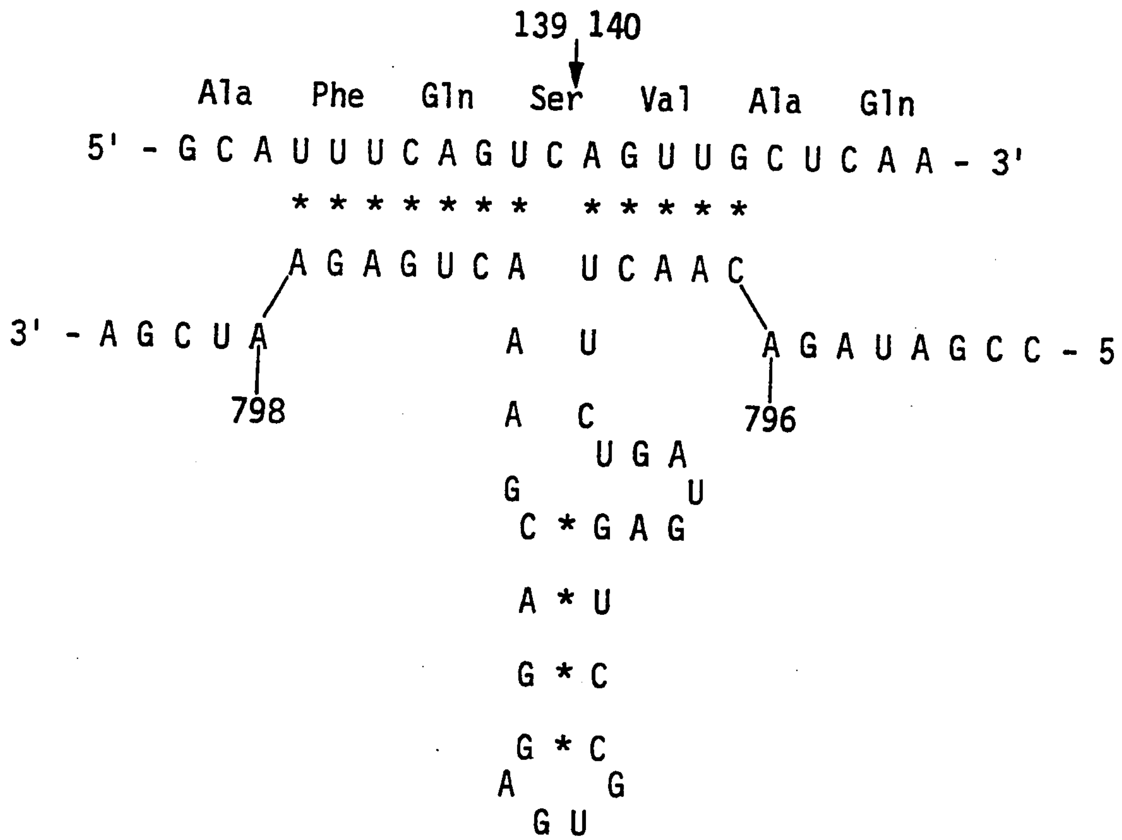


FIG. 1A

FIG. 1B

FIG. 1C


139 140
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A G A G U C A U C A A C
3' - A G C U A
A G A U A G C C - 5'
798
796
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FIG. 2

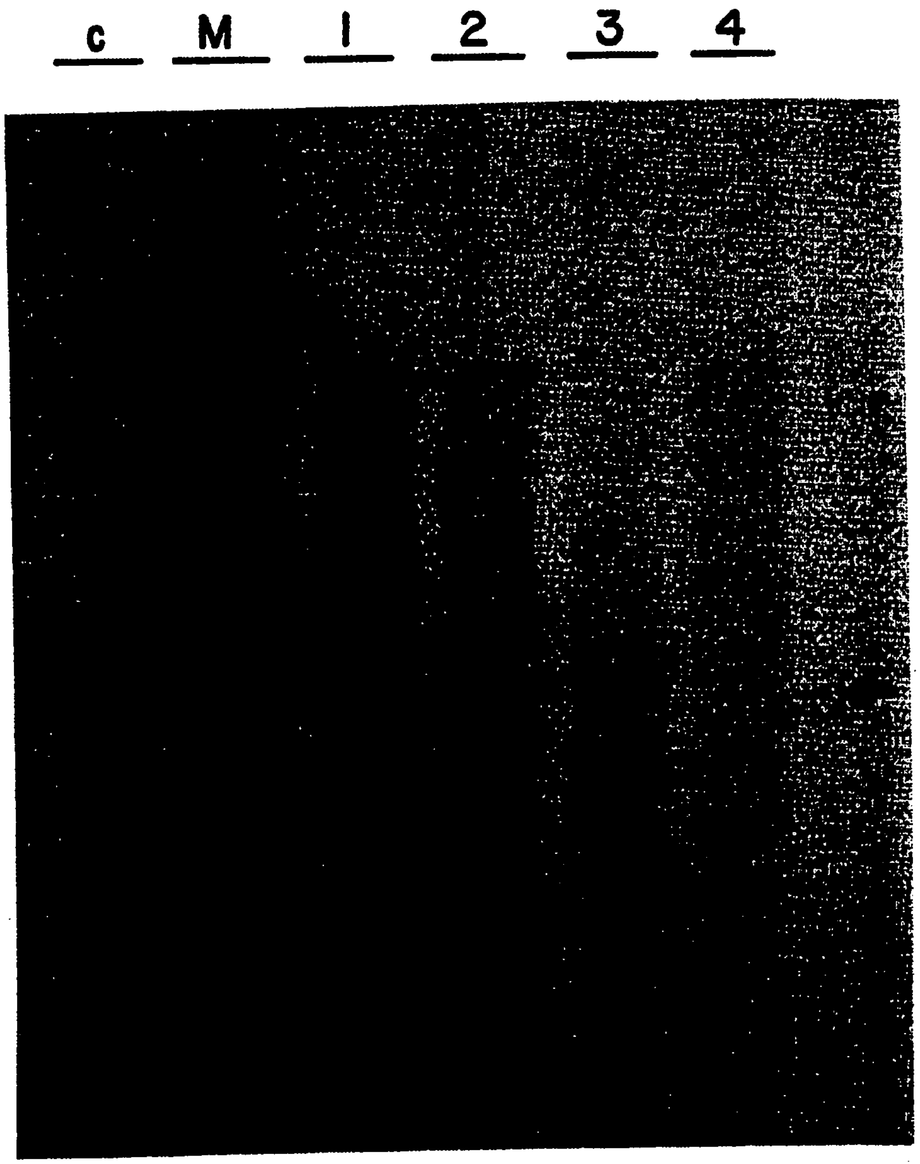


FIG. 3

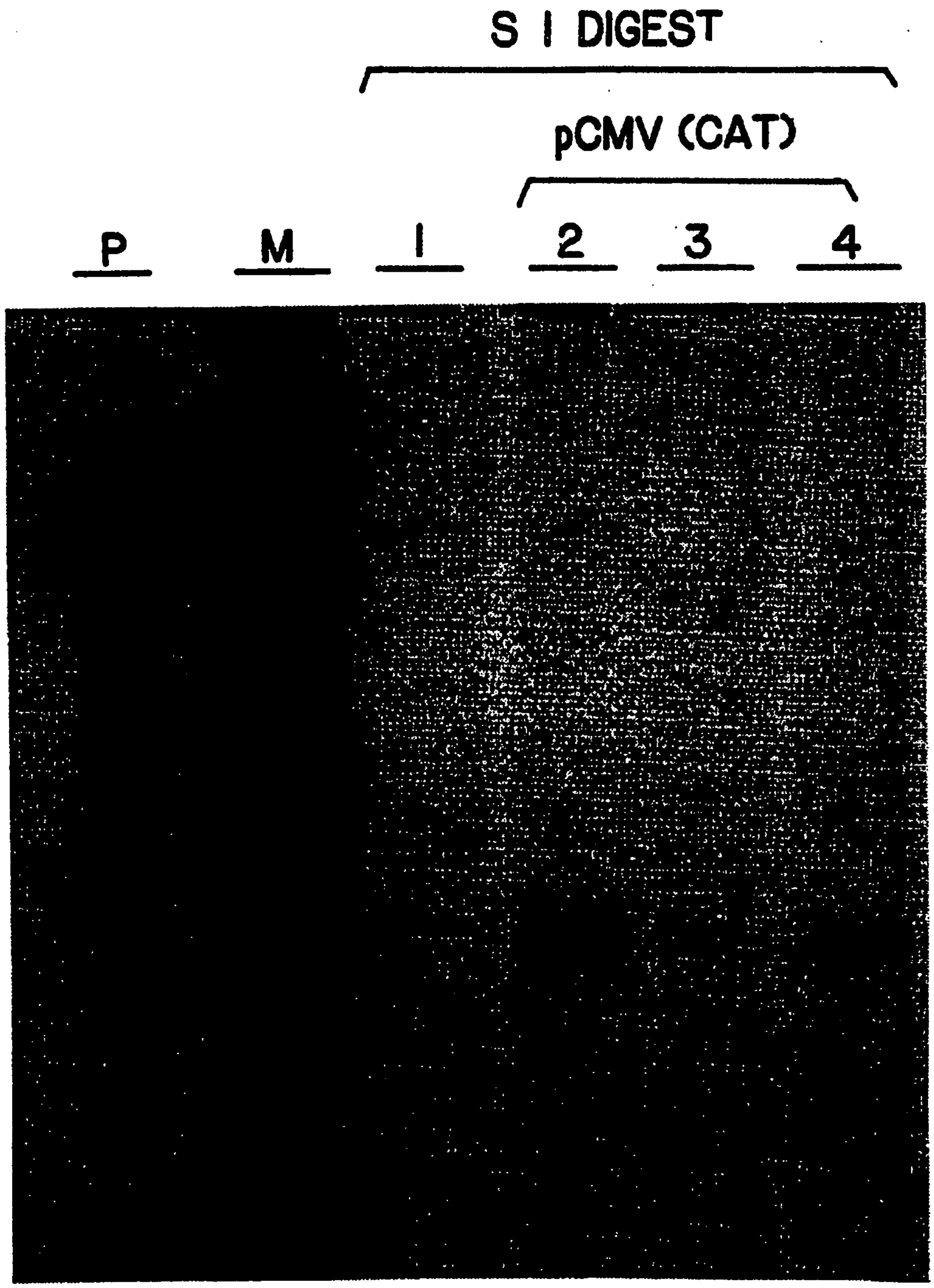
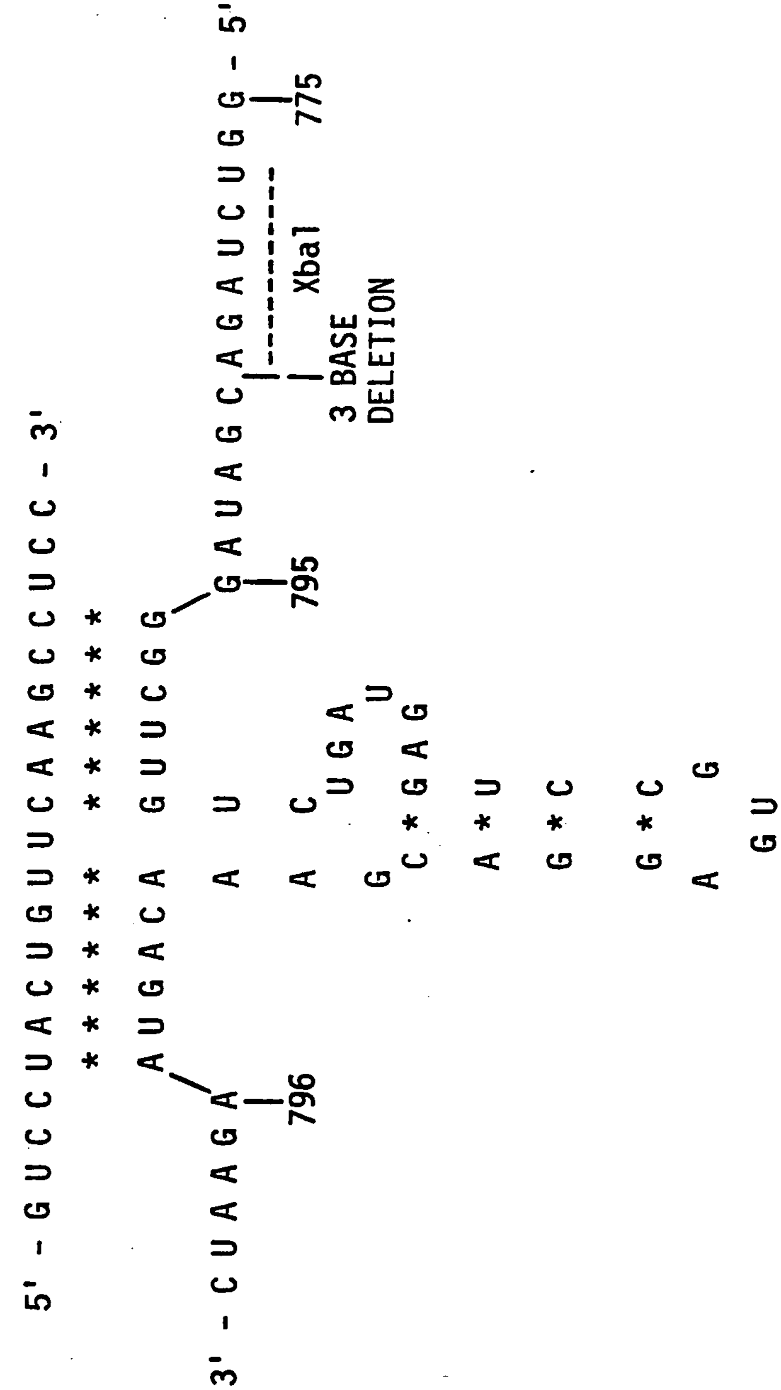


FIG. 4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3' - CUAAGA
GAUAGCAGAUCUGG - 5'
796
795
775
XbaI
3 BASE
DELETION

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07859

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 31/70, 35/76; C12N 15/00, 15/11, 15/36
US CL : Please See Attached Sheet.

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	514/54; 536/27; 435/320.1, 172.1; 935/14, 22, 27, 32, 39, 42, 56, 57, 62, 65

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Biosis, Medline, Aps

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X	US, A, 4,942,125 (Moriarty) 17 July 1990, see the entire document, especially columns 15-16.	1-4
Y	Journal of Virology, Volume 63, No. 5, issued May 1989, Kuo et al, "Initiation of Replication of the Heman Hepatitis Delta Virus Genome from Cloned DNA: Role of Delta Antigen", pages 1945-1950, see the entire document.	1-23
Y	Proc. Natl. Acad. Sci. USA, Vol. 86, issued March 1989, Wu et al "Human hepatitis δ virus RNA subfragments contain an autocleavage activity", pages 1831-1835, see the entire document.	1-23
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Y	Proc. Natl. Acad. Sci. USA, Volume 86, issued 1989, Cameron et al, "Specific gene suppression by engineered ribozymes in monkey cells", pages 9139-9143, see the entire document.	1-23

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
30 JANUARY 1992	18 Feb 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	Jeanne Mann for Jacqueline M. Stone

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/54; 536/27; 435/320.1, 172.1; 935/14, 22, 27, 32, 39, 42, 56, 57, 62, 65